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Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M511542200

Publication date:
2006

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 10, pp. 6482–6488, March 10, 2006

Carbonic Anhydrase Subunits Form a Matrix-exposed Domain Attached to the Membrane Arm of Mitochondrial Complex I in Plants*[S]

Received for publication, October 25, 2005, and in revised form, December 30, 2005 Published, JBC Papers in Press, January 4, 2006, DOI 10.1074/jbc.M511542200

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Complex I of Arabidopsis includes five structurally related subunits representing γ-type carbonic anhydrases termed CA1, CA2, CA3, CAL1, and CAL2. The position of these subunits within complex I was investigated. Direct analysis of isolated subcomplexes of complex I by liquid chromatography linked to tandem mass spectrometry allowed the assignment of the CA subunits to the membrane arm of complex I. Carbonate extraction experiments revealed that CA2 is an integral membrane protein that is protected upon protease treatment of isolated mitoplasts, indicating a location on the matrix-exposed side of the complex. A structural characterization by single particle electron microscopy of complex I from the green alga Polytomella and a previous analysis from Arabidopsis indicate a plant-specific spherical extra-domain of about 60 Å in diameter, which is attached to the central part of the membrane arm of complex I on its matrix face. This spherical domain is proposed to contain a heterotrimer of three CA subunits, which are anchored with their C termini to the hydrophobic arm of complex I. Functional implications of the complex I-integrated CA subunits are discussed.

Carbonic anhydrases (CA)[2] are zinc-containing enzymes that catalyze the reversible interconversion of CO_2 and HCO_3^-. In higher plants, carbonic anhydrases have been localized to the chloroplast stroma and to the cytoplasm; in algae additionally to the mitochondrion (reviewed in Moroney *et al.*, Ref. 1)). Chloroplast carbonic anhydrases are important for efficient delivery of CO_2 to ribulose-bisphosphate carboxylase/oxygenase (RubisCO). In contrast, the physiological role of carbonic anhydrases of other subcellular compartments is still a matter of debate. Structurally, carbonic anhydrases can be divided into three families termed α, β, and γ, which evolved independently (2). The chloroplast carbonic anhydrases belong to the β family. Homologs of carbonic anhydrases from all three families were identified in the course of the Arabidopsis genome sequencing project (1, 3), but so far have not been physiologically characterized.

Recently, γ-type carbonic anhydrases were found to be localized within plant mitochondria attached to complex I of the respiratory chain. This complex was first purified about 10 years ago from plants (Refs. 4–8, reviewed in Rasmusson *et al.* Ref. 9). Many of the characterized subunits were found to be homologous to subunits of complex I from fungi and animals, but some did not exhibit any significant sequence identity, *e.g.* an "unknown 29-kDa protein" of potato complex I (5). Systematic identification of complex I proteins in Arabidopsis, rice, and Chlamydomonas led to the discovery of up to 5 subunits related to the "unknown 29-kDa protein" within this respiratory complex of these organisms (10, 11).[3] Based on sequence comparisons, these subunits were initially proposed to be called "ferripyochelin-binding proteins." However, later it became clear that these identifications were based on a falsely annotated data base entry (12). Instead, significant sequence identity was discovered to the prototype γ-carbonic anhydrase of the Archaebacterium *Methanosarcina thermophila* (12). Computer modeling using the crystal structure of the archaebacterial γ-carbonic anhydrase revealed that at least three of the five plant-specific complex I subunits of Arabidopsis have a conserved active site (12). They are called carbonic anhydrase 1 (CA1) (At1g19580), CA2 (At1g47260), and CA3 (At5g66510) in Arabidopsis. Two further related complex I subunits of Arabidopsis have a less conserved primary structure and are termed carbonic anhydrase-like protein 1 (CAL1) (At5g63510) and CAL2 (At3g48680) (13).

The physiological role of CA2 and CA3 in plant mitochondria was addressed with the use of Arabidopsis knock-out lines (14). Surprisingly, the phenotype of the mutants was not distinguishable from a wild-type line under all conditions tested, which might be a consequence of redundant activities of the five related complex I subunits. However, suspension cell cultures of the knock-out lines had a reduced growth rate. Furthermore, complex I levels were clearly reduced in mutant lines, indicating that the CA subunits are important for complex I assembly. The genes encoding CA1 and CA2 were found to be down-regulated when Arabidopsis plants are grown under elevated CO_2 concentrations, supporting a role of these proteins in mitochondrial one-carbon metabolism. Possibly the CA subunits play important roles in the context of photorespiration, which leads to the liberation of large amounts of CO_2 in plant mitochondria, especially under high light conditions (14).

* This work was supported by Grant Br1829-7/1 from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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[2] The abbreviations used are: CA, carbonic anhydrase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MOPS, 4-morpholinepropanesulfonic acid; EM, electron microscopy; ANT, adenine nucleotide translocator; SOD, superoxide dismutase; PMSF, phenylmethylsulfonyl fluoride.

[3] H. P. Braun, unpublished results.

Supplemental Material can be found at: http://www.jbc.org/cgi/content/full/M511542200/DC1

The location of the carbonic anhydrase subunits within mitochondrial complex I so far is unknown. The CAL1 and CAL2 subunits of Arabidopsis were shown to interact with CA2 using the yeast two-hybrid system (13). Very recently, the projection structure of Arabidopsis complex I was resolved by electron microscopy and single particle analysis (15). Interestingly, it shows an extra matrix-exposed domain, which is attached to the membrane arm of this complex. Here we present evidence that CA2 forms part of this extra-domain: CA2 cannot be detached from mitochondrial membranes by carbonate extraction, was identified by mass spectrometry to form part of the membrane arm of complex I and is shown to be localized on the matrix-exposed side of this arm by protease protection experiments with isolated mitoplasts. Location of the carbonic anhydrase domain of complex I was confirmed by a structural analysis of this complex from Polytomella by electron microscopy and single particle analysis.

MATERIALS AND METHODS

Cultivation of Arabidopsis and Polytomella—Arabidopsis cell suspension cultures were established as described by May and Leaver (16). Cells were cultivated in 500-ml flasks containing 100 ml of medium (0.316% (w/v) B5 medium, 3% (w/v) sucrose, 0.0001% (w/v) 2,4 dichlorphenoxyacetic acid, 0.00001% (w/v) kinetin, pH 5.75) at 25 °C, and 90 rpm in the dark. Cells were transferred into fresh medium all 7 days. The starting material for mitochondrial isolation was ~100 × *g* cells.

Polytomella spp. (198.80, E. G. Pringsheim) was obtained from the "Sammlung von Algenkulturen der Universität Göttingen" (SAG) (epsag.uni-goettingen.de/html/sag.html). Cells were cultivated in 2.5-liter culture flasks containing 1200 ml of medium (0.2% (w/v) sodium acetate, 0.1% (w/v) yeast extract, 0.1% (w/v) tryptone) for 4–5 days at 25 °C in the dark without shaking. The starting material for mitochondrial isolations was ~100 × *g* cells.

Isolation of Mitochondria from Polytomella—Mitochondria from Polytomella were purified as outlined in Dudkina *et al.* (17). Organelles were resuspended in resuspension buffer (0.4 M mannitol, 1 mM EGTA, 10 mM Tricine, 0.1 mM PMSF, pH 7.2) at a protein concentration of 10 mg/ml, divided into 100-μl aliquots, and stored at −80 °C.

Isolation and Subfractionation of Mitochondria from Arabidopsis—Mitochondria from Arabidopsis were purified as outlined in Werhahn *et al.* (18). Organelles were resuspended in resuspension buffer (0.4 M mannitol, 1 mM EGTA, 10 mM Tricine, 0.2 mM PMSF, pH 7.2) and either stored at −80 °C or directly used for the generation of mitochondrial subfractions. In the latter case, mitochondria were sedimented by centrifugation for 10 min at 12 000 × *g* and resuspended in resuspension buffer without mannitol at a protein concentration of 10 mg/ml. Mitochondria were broken by sonication with a Ultrasonic cell disruptor (Misonix Inc.) in four intervals of 10 s. Unbroken mitochondria were sedimented by centrifugation for 7 min at 5000 × *g* and discarded. The supernatant was centrifuged at 150,000 × *g* for 90 min. The resulting pellet contains the mitochondrial membranes whereas the supernatant contains the soluble proteins of the mitochondrial matrix and the intermembrane space. The pellet was resuspended with resuspension buffer at a protein concentration of 10 mg/ml. Membrane and soluble fractions were divided into aliquots and stored at −80 °C. The purity of subfractions was analyzed by two-dimensional blue-native PAGE (see below) or by immunoblotting experiments using antibodies directed against marker proteins of the mitochondrial membrane and soluble fractions (see "Results").

Carbonate Extraction—For carbonate extraction, mitochondrial membranes were resuspended in carbonate buffer (0.1 M Na_2CO_3, 0.1 mM PMSF, pH 11.5) at a protein concentration of 10 mg/ml and incubated for 5 min at 4 °C. Afterward the membranes were again sedimented by ultracentrifugation for 90 min at 150,000 × *g*. The pellet was finally resuspended in resuspension buffer at a protein concentration of 10 mg/ml and divided into aliquots (membranes with integral membrane proteins); the supernatant was directly divided into aliquots (peripheral membrane proteins). All aliquots were stored at −80 °C.

Protease Protection Experiments—Mitoplasts (mitochondria lacking the outer membrane) were generated from isolated mitochondria to obtain topological information on inner membrane proteins: first mitochondria were sedimented by centrifugation and resuspended in swelling buffer (5 mM potassium phosphate, pH 7.2) at a protein concentration of 5 mg/ml. Subsequently the outer mitochondrial membrane was selectively ruptured by treatment with a Teflon homogenizer (20 strokes). Resulting mitoplasts and outer membrane fragments were next separated by sucrose gradient ultracentrifugation (step gradient of 60%, 32%, and 15% sucrose in 1 mM EDTA, 10 mM MOPS, pH 7.2) for 1 h at 92,000 × *g*. Mitoplasts are enriched at the 32/60% interphase of the gradients. They were purified from sucrose by dilution with resuspension buffer, sedimentation by centrifugation for 10 min at 12,000 × *g*, and again resuspended in resuspension buffer at a protein concentration of 10 mg/ml. For protease protection experiments, 250-μl mitoplasts were combined with 10 μl of protease solution (0.1% proteinase K in 10 mM Tris-HCl, pH 7.2) and incubated for 30 min on ice. The reaction is stopped by addition of 2.5 μl of PMSF solution (200 mM PMSF). Fractions were either directly analyzed by one-dimensional SDS-PAGE and immunoblotting (see below) or stored at −80 °C.

Gel Electrophoresis Procedures and Immunoblotting—One-dimensional SDS-PAGE was carried out according to Schägger *et al.* (19), one-dimensional blue-native PAGE and two-dimensional blue-native/SDS-PAGE according to Heinemeyer *et al.* (20), and two-dimensional blue-native/blue-native PAGE as outlined by Sunderhaus *et al.* (21). Proteins were either visualized by Coomassie Blue-colloidal staining (22, 23), in-gel NADH dehydrogenase activity staining (24), or blotted onto nitrocellulose filters. Blots were incubated overnight with different antibodies directed against the mitochondrial adenine nucleotide translocator (ANT), the mitochondrial superoxide dismutase (SOD), and the C-terminal half of the mitochondrial CA (At1g47260) from Arabidopsis (14). Visualization of immune-positive bands was performed using biotinylated secondary antibodies, avidin, and horseradish peroxidase (Vectastain ABC kit, Vector Labs). Selected protein complexes were cut out from Coomassie Blue-stained two-dimensional blue-native/blue-native gels and subunits were identified by mass spectrometry (25).

Purification of Complex I from Polytomella—Complex I from Polytomella was purified as outlined in Dudkina *et al.* (15) for the corresponding protein complex from Arabidopsis. About 1 mg of Polytomella mitochondria (100 μg of mitochondrial protein) was redissolved in digitonin solubilization buffer (5.0% digitonin, 30 mM HEPES, 150 mM potassium acetate, pH 7.4). Solubilized protein complexes were subsequently separated by sucrose gradient ultracentrifugation (gradients of 0.3–1.5 M sucrose/15 mM Tris base, pH 7.0, 20 mM KCl/0.2% digitonin) for 20 h at 150,000 × *g*. Fractions were removed from the gradient from bottom to top. The protein complex content of the fractions was analyzed by one-dimensional blue-native PAGE and two-dimensional blue-native/SDS-PAGE (see above). Complex I-containing fractions were used for EM analyses.

Electron Microscopy and Image Analysis—Negatively stained specimens were prepared with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Images were recorded with a Gatan 4K slow-scan CCD camera on a Philips CM12 electron microscope. 2000 × 2000 pixel images were recorded at ×77,400 magnification with a pixel size of 15

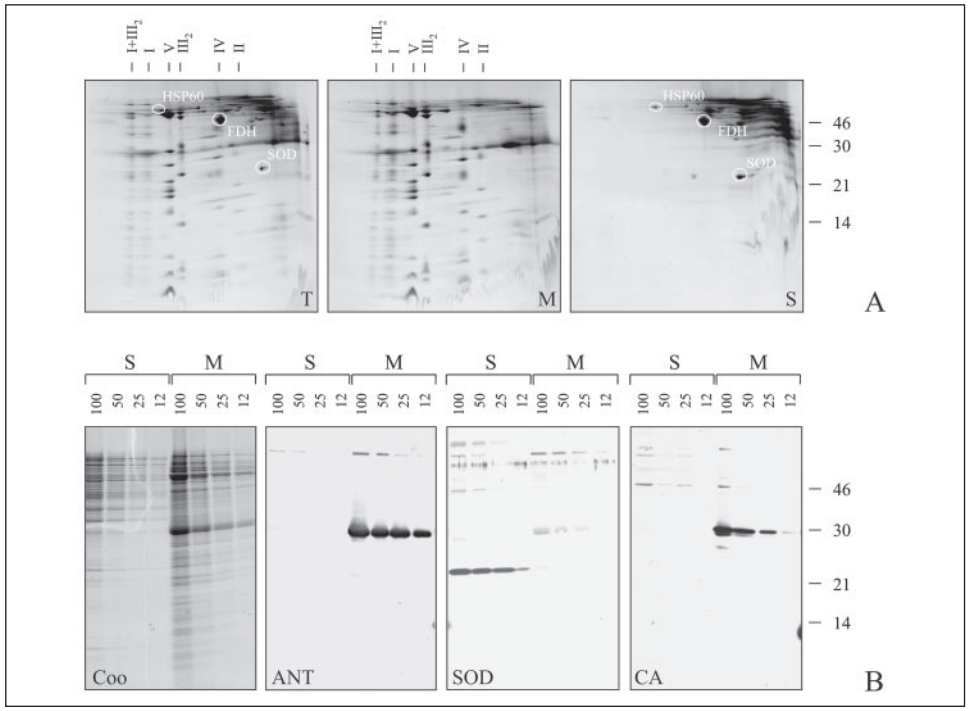

FIGURE 1. **Submitochondrial localization of the carbonic anhydrase CA2.** Mitochondria were isolated and subfractionated into a membrane and a soluble fraction as described under "Materials and Methods." *A*, documentation of the purity of the subfractions by two-dimensional blue-native/SDS-PAGE. *T*, total mitochondrial protein; *M*, protein of the membrane fraction; *S*, protein of the soluble fraction. Identities of membrane-bound protein complexes are given above the gels (*I*, complex I; *II*, complex II; III_2, dimeric complex III; *IV*, complex IV; *V*, ATP synthase complex; $I+III_2$, supercomplex formed by complex I and dimeric complex III) and identities of soluble protein complexes directly on the two-dimensional gels (*HSP60*, heat stress protein *60*, *FDH*, formate dehydrogenase). The molecular masses of standard proteins are given to the *right* (in kDa). *B*, immunological localization of carbonic anhydrase. Varying amounts of protein (indicated *above* the gels in μg) of the soluble (*S*) and the membrane (*M*) fraction were resolved by one-dimensional SDS-PAGE and Coomassie-stained (*Coo*) or blotted onto nitrocellulose. Proteins of interest were detected using antibodies directed against ANT (membrane marker), SOD (matrix marker), and CA. Molecular masses of standard proteins are given to the *right* of the gels (in kDa).

μm and a binning factor of 2, corresponding to a size of 3.85 Å at the specimen level. Single particle projections were extracted from images and analyzed with Groningen image processing (Grip) software on a PC cluster. Images were subsequently subjected to multireference alignment, multivariate statistical analysis, and hierarchical classification as described before (15). Resolution of averaged single particle two-dimensional projections was measured according to van Heel (26).

RESULTS

The Carbonic Anhydrase CA2 of Arabidopsis Is an Integral Membrane Protein—Complex I of plants purified by chromatographic or electrophoretic procedures was shown to include carbonic anhydrase subunits. Deletion of the gene encoding CA2 drastically reduces complex I levels in Arabidopsis, indicating an integral position of this protein within this respiratory complex. However, a more loosely binding of the carbonic anhydrase subunits to complex I so far cannot be excluded. Therefore, localization of the carbonic anhydrases was immunologically tested using submitochondrial fractions. The following results are based on an antibody directed against the C-terminal half of CA2 (At1g47260), which previously was shown to be monospecific for the CA2 protein (14).

Mitochondria from Arabidopsis were subfractionated into a membrane (M) and a soluble (S) fraction as described under "Materials and Methods." The purity of the generated fractions was tested by two-dimensional blue-native PAGE (Fig. 1*A*). The respiratory protein complexes, which were identified on the basis of their subunit compositions (27), were exclusively present in the membrane fraction. In contrast the formate dehydrogenase complex, the HSP60 complex and the SOD of the mitochondrial matrix were absent in this fraction but present in the soluble fraction. The purity of the mitochondrial subfractions was confirmed by one-dimensional SDS-PAGE and immunoblotting using antibodies directed against ANT (marker for the mitochondrial membrane fraction) and SOD (marker for the soluble mitochondrial fraction) (Fig. 1*B*). ANT exclusively was recognized in the membrane fraction, SOD in the soluble fraction. In conclusion, the generated subfractions can be considered to be very pure.

In a parallel immunoblotting experiment, CA2 was only detectable in the membrane fraction. Probing the 12-μg sample of the membrane fraction gave a clear signal on the immunoblot, whereas no signal was observed in the corresponding 100-μg sample of the soluble fraction (Fig. 1*B*, *right immunoblot*). This suggests that CA2 is a membrane protein.

In the next step, carbonate extraction was employed to test whether CA2 is an integral membrane protein or associated to the surface of the membrane. Mitochondria were isolated from Arabidopsis and subfractionated into a soluble fraction (SP), a fraction containing peripheral membrane proteins (PMP), and a fraction containing integral membrane proteins (IMP) as described under "Materials and Methods." All three subfractions have a very distinct composition of proteins as monitored by one-dimensional SDS-PAGE in combination with Coomassie Blue-staining (Fig. 2). Parallel immunoblotting experiments were carried out to monitor the purity of the generated subfractions. As expected, only SOD is present in the soluble fraction and ANT in the fraction of integral membrane proteins. Similarly, CA2 was nearly exclusively present in the fraction of integral membrane proteins. We conclude that CA2 is an integral membrane protein.

The Carbonic Anhydrase CA2 Forms Part of the Membrane Arm of Complex I—Complex I has an L-like shape. The matrix arm, which is responsible for NADH oxidation, protrudes into the matrix. It is attached to a membrane arm responsible for the proton translocation activity of complex I. The location of CA2 with respect to these two arms was investigated by blue-native/blue-native PAGE in combination with in-gel NADH oxidation activity staining and mass spectrometry. Proteins normally are localized on a diagonal line on gel systems if the same buffer and detergent conditions were used for both dimensions. However, in an alternative approach of blue-native/blue-native PAGE, the first gel dimension is carried out in the presence of digitonin, which is for the most part mild for protein solubilization, and the second gel dimension in the presence of dodecylmaltoside, which is slightly less mild (28). Protein complexes specifically destabilized in the presence of the second detergent were divided on this two-dimensional gel system into subcomplexes of enhanced electrophoretic mobility, which are vis-

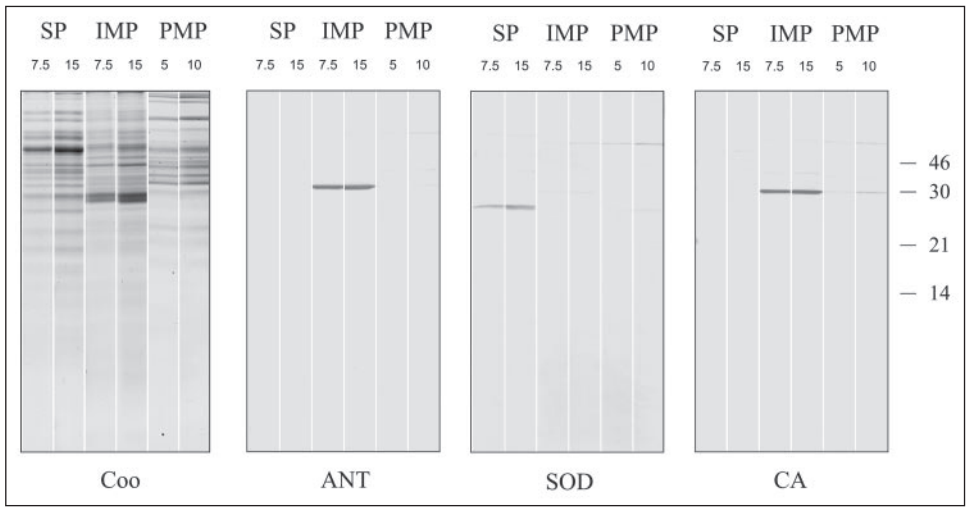

FIGURE 2. **Analysis of the anchoring of carbonic anhydrase CA2 within the inner mitochondrial membrane by carbonate extraction.** Peripheral and integral membrane proteins were separated by carbonate treatment and subsequent centrifugation as described under "Materials and Methods." Two different protein amounts (indicated *above* the gels in μg) of fractions containing soluble mitochondrial proteins (*SP*), integral membrane proteins (*IMP*), and peripheral membrane proteins (*PMP*) were separated by one-dimensional SDS-PAGE and Coomassie Blue-stained (*Coo*) or blotted onto nitrocellulose. Proteins of interest were immunodetected using antibodies directed against SOD (matrix marker), ANT (membrane marker), and CA. The molecular masses of standard proteins are given to the *left* of the Coomassie Blue-stained gel.

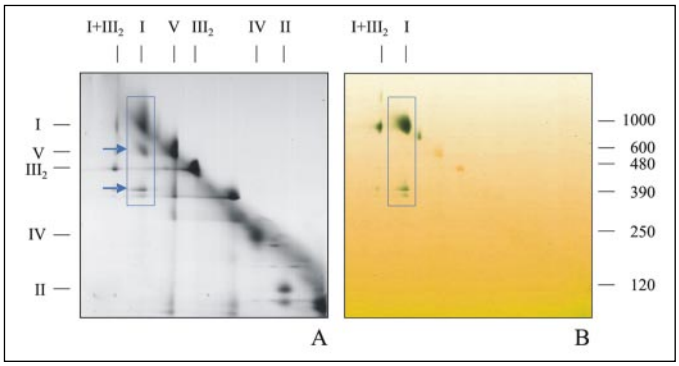

FIGURE 3. **Partial division of complex I from Arabidopsis into two subcomplexes.** Total mitochondrial membrane protein of Arabidopsis was resolved by two-dimensional blue-native/blue-native PAGE. First dimension blue-native PAGE was carried out in the presence of digitonin, second dimension blue-native PAGE in the presence of dodecylmaltoside. *A*, Coomassie Blue-stained two-dimensional gel; *B*, complex I activity-stained gel (NADH oxidation). Identities of protein complexes are given on *top* of the gels and to the *left* of the Coomassie Blue-stained gel; molecular masses of standard protein complexes are given to the *right* of the activity-stained gel. Complex I and its subcomplexes of 600 and 400 kDa (*arrows*) are marked by a *box*.

ible beneath the diagonal line. Using this gel system, complex I of Arabidopsis (1000 kDa) partially becomes divided into a 600- and a 400-kDa subcomplex (Fig. 3). For unknown reasons, the 400-kDa complex in part gets further divided into a 380-kDa complex. The 400/380-kDa complexes represent the matrix arm of complex I as shown by in-gel NADH oxidation activity staining (Fig. 3). We conclude that the 600-kDa complex must represent the membrane arm.

To localize CA2 within complex I, the two 600- and 400-kDa subcomplexes were directly cut out from the two-dimensional blue-native/blue-native gel, trypsinated, and analyzed by liquid chromatography in combination with tandem mass spectrometry (Table 1). 19 different proteins could be identified forming part of the 600-kDa complex, 16 of which were previously known (Ref. 10 and references within) and 3 of which were described for the first time (At1g14450, At4g00585, At1g67350). As expected, several of the subunits forming part of the 600-kDa subcomplex of complex I from Arabidopsis are homologous to complex I subunits from beef and *Neurospora crassa* known to be present in the membrane arm. Furthermore, 4 of the 5 CA subunits (γCA1, γCA2, γCA3, γCAL2) were identified forming part of the 600-kDa complex. In contrast, no CA subunits were identified within the 400-kDa subcomplex, which includes 4 known proteins of the matrix arm (Table 1). We conclude that the CA subunits of complex I from Arabidopsis are integral membrane proteins, which form part of the membrane arm of this complex. This result was confirmed by immunoblotting experiments using two-dimensional blue-native/blue-native gels (data not shown).

The Carbonic Anhydrase CA2 Is Localized on the Matrix-exposed Side of the Membrane Arm of Complex I—The topological localization of CA2 within the membrane arm of complex I was addressed by protease protection experiments using isolated mitoplasts (mitochondria lacking the outer mitochondrial membrane). Mitoplasts of Arabidopsis were incubated with proteinase K as outlined under "Materials and Methods." Subsequently, proteins of protease-treated and untreated mitoplasts were analyzed by SDS-PAGE and immunoblotting (Fig. 4). As expected, SOD was protease-protected, because it was localized within the mitochondrial matrix. ANT was accessible to protease digestion. As known from the crystal structure of this protein from beef, ANT is composed of six membrane-spanning helices (29). The N and the C terminus as well as two internal loops are exposed to the intermembrane space. Therefore, degradation of ANT upon protease treatment of mitoplasts into several distinct fragments is expected. In contrast, the 30-kDa CA2 proved to be protease-protected. Only one very faint degradation product was visible on the corresponding immunoblot at 28 kDa (Fig. 4). We conclude that CA2 is an integral membrane protein of the membrane arm of complex I, which is localized on the matrix side of this arm. Possibly a small part (2 kDa) of the protein protrudes into the intermembrane space.

Polytomella Also Has the Matrix-exposed Extra-domain of Complex I—Single particle electron microscopy is a suitable technique for a direct structural characterization of CA subunits within complex I. Previously, EM data from Arabidopsis revealed a spherical extra matrix-exposed domain, which is attached to the central part of the membrane arm (15). Most likely the extra-domain represents the carbonic anhydrase subunits of complex I. This hypothesis was tested with an alga species. The green alga Chlamydomonas was recently reported to also include homologous CA subunits within complex I (11). It therefore should also have an extra CA domain. This was investigated using purified mitochondria from *Polytomella spp.*, a non-green alga of the Chlamydomonaceae closely related to *Chlamydomonas reinhardtii*. Analysis of the mitochondria by two-dimensional blue-native/SDS-PAGE allowed us to identify the known respiratory protein complexes of Polytomella (30–32): dimeric complexes III and V, two forms of complex IV, and complex I. Complex I is partially divided into a smaller

TABLE 1
Identified subunits of the 600- and 400-kDa subcomplexes of complex I

Protein complex[a]	Accession numbers[b]	Proteins[c]	Calc mol. mass[d]	MOWSE score[e]	Coverage[f]
600 kDa	At1g47260	NADH-DH carbonic anhydrase subunit	30.0	391	13
	At3g48680	NADH-DH carbonic anhydrase subunit	28.0	153	5
	At2g33220	NADH-DH B 16.6 subunit (*N. crassa*)	16.1	124	6
	At1g04630	NADH-DH B 16.6 subunit (*N. crassa*)	16.1	124	6
	At2g27730	NADH-DH 16 kDa (*Solanum tuberosum*)	11.9	123	2
	At5g66510	NADH-DH carbonic anhydrase subunit	27.8	105	4
	At2g02050	NADH-DH CI-B18 (beef)	11.7	88	5
	At1g14450	Unknown protein	8.2	85	3
	At1g19580	NADH-DH carbonic anhydrase subunit	30.0	84	4
	At4g34700	NADH-DH chain CI-B22 (*N. crassa*)	13.6	73	3
	At4g16450	NADH-DH 20.9-kDa chain (*N. crassa*)	11.4	73	3
	At1g67350	Unknown protein	15.2	59	3
	At2g31490	NADH-DH subunit (*Arabidopsis thaliana*)	8.2	57	2
	At3g18410	NADH-DH 14-kDa subunit (*S. tuberosum*)	12.4	56	1
	At1g49140	NADH-DH PDSW subunit (beef)	12.5	56	1
	At2g47690	NADH-DH 15-kDa subunit (beef)	14.0	55	2
	AtMg01120	NADH-DH, subunit 1	36.0	49	1
	At4g00585	Unknown protein	9.8	49	1
	At2g42310	NADH-DH subunit (*A. thaliana*)	12.6	41	2
400 kDa	At5g37510	NADH-DH, 76-kDa chain precursor protein	81.5	187	6
	At2g20360	NADH-DH, 40-kDa chain (*N. crassa*) = 39-kDa protein	43.9	104	3
	nad7	NADH-DH, subunit 7 = nuoD	44.6	72	2
	At5g52840	NADH-DH 22.5-kDa subunit (*S. tuberosum*) 13-kDa-B subunit (B13) (beef)	19.2	32	2

[a] Protein complexes were directly excised from the Coomassie Blue-stained BN / BN gel shown in Fig. 3.
[b] Accession numbers corresponding to TAIR (www.arabidopsis.org/).
[c] Protein identities based on sequence identity to complex I subunits from other organisms as revealed by BLAST searches.
[d] Calculated molecular mass of the identified precursor proteins (in kDa).
[e] MOWSE score from the MASCOT software package. (www.matrixscience.com/).
[f] Number of matching peptides.

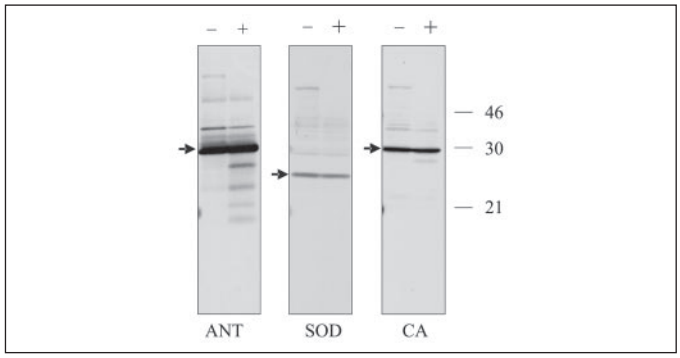

FIGURE 4. **Topographical analysis of carbonic anhydrase CA2 by protease protection experiments.** Mitoplasts (mitochondria lacking the outer membrane) were incubated with proteinase K as described under "Materials and Methods." Total mitochondrial protein of untreated (−) and treated (+) fractions was resolved by SDS-PAGE and blotted onto nitrocellulose. Proteins of interest were identified by immunodetection using antibodies directed against ANT (marker for an integral inner membrane protein accessible for protease digestion), SOD (marker for a matrix protein inaccessible for protease digestion), and CA.

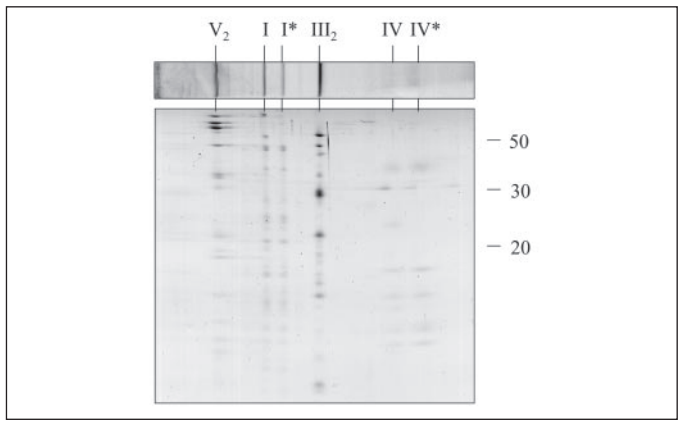

FIGURE 5. **Two-dimensional resolution of mitochondrial protein complexes from Polytomella.** Isolated mitochondria from Polytomella were solubilized with 5% digitonin as described under "Materials and Methods." Proteins were separated by one-dimensional blue-native PAGE (gel stripe on *top*) and subunits of the protein complexes by a second gel dimension in the presence of SDS. Molecular masses of standard proteins are given to the *right* of the gel and identities of protein complexes on *top*. V_2, dimeric ATP synthase; *I*, complex I; *I**, subcomplex of complex I lacking subunits of the NADH oxidizing domain; III_2, dimeric complex III; *IV*, complex IV; *IV**, subcomplex of complex IV.

form, which lacks the large subunits of the NADH oxidation domain (*complex I** in Fig. 5).

For EM analysis of complex I, mitochondrial protein complexes were solubilized using digitonin and separated by sucrose density ultracentrifugation. Complex I-containing fractions of the gradient were directly analyzed by EM in combination with single particle analysis (Fig. 6). A subset of 6,500 side view projections was analyzed. Four different projection views could be distinguished, which represent the larger and the smaller form of complex I from both sides, respectively (Fig. 6, *panels a*, *b*, *d*, and *e*). In these views the stronger stain-excluding membrane-bound arm with a length of 210 Å runs horizontal and the hydrophilic arm almost vertical. The smaller particle, which lacks part of the matrix arm (Fig. 6, *panels a* and *b*) is more abundant than the intact particle (Fig. 6, *panels d* and *e*). A fifth projection class represents a top view of the Polytomella complex I (Fig. 6*c*). From this perspective, the membrane arm is slightly bent, which previously was reported for the Arabidopsis supercomplex consisting of complex I and dimeric complex III (Fig. 6, *panel f* and Ref. 15). The best projection maps of Fig. 6, *panels b* and *c* have a resolution of about 17 Å.

All the side view projections of Polytomella complex I show the matrix-exposed extra-domain at the center of the membrane arm of the complex (Fig. 6, *panels a–f*, *white arrows*). The side views indicate that this spherical domain has a diameter of about 60 Å in both Polytomella (Fig. 6, *panels a* and *e*) and Arabidopsis (Fig. 6, *panels g* and *h*). In our hypothesis it contains a heterotrimer of different carbonic anhydrase subunits, as discussed below. To verify this hypothesis we did an additional single particle electron microscopy study on purified complex I

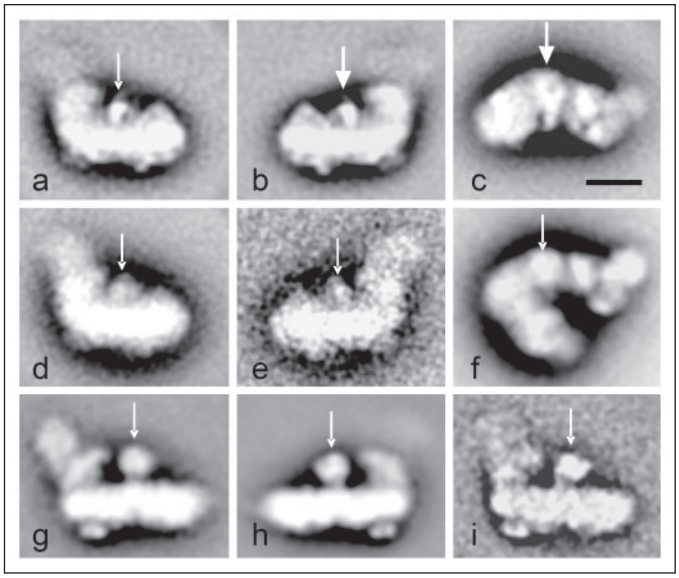

FIGURE 6. **Structure of complex I from Polytomella spp.** A data set of 11,000 single particle projections was analyzed by multireference alignment and multivariate statistical analysis. A total of 6581 projections could be assigned to side views of the larger and smaller form of complex I in two different positions, respectively (*panel a*, average of 1449 projections; *panel b*, average of 4608 projections; *panel d*, average of 512 projections; *panel e*, average of 16 projections). 1366 projections represent a *top* view (*panel c*). *Panel f*, averaged projection map of top view projections of I-III$_2$ supercomplex particles from Arabidopsis; *panels g* and *h*, average of side view projections of a complete complex I and complex I lacking a part of the NADH-oxidizing domain from Arabidopsis (taken from Dudkina *et al.* (15), Fig. 2, *panels d* and *e*); *panel i*, total sum of 318 projections of complex I in a side view position from an Arabidopsis mutant lacking the At1g47260 gene encoding CA2. The plant-specific carbonic anhydrase domain is indicated by an *arrow*. The *bar* equals 10 nm.

from an Arabidopsis knock-out mutant of gene At1g47260, encoding the CA2 subunit. The total sum of 318 projections represents a complex I particle, which is very similar or identical to the wild type (Fig. 6, *panel i*). Most likely, other structurally related CA subunits of complex I are compensating for the absence of CA2.

DISCUSSION

The subunit composition of complex I is well known for several organisms (33–35). In eukaryotes, 40–45 subunits form part of this complex, many of which have a conserved primary structure in fungi and animals. In contrast, complex I from plants includes quite a number of plant-specific subunits, most strikingly a group of 3–5 structurally related γ-carbonic anhydrases (10, 11). Using a monospecific antibody directed against CA2, this protein was shown to be an integral membrane protein, which is attached to the membrane arm of complex I on its matrix side. These results nicely fit to a special structural feature of plant complex I. In contrast to bacteria, fungi, and animals, Arabidopsis complex I has an extra spherical matrix-exposed domain attached to the central part of its membrane arm (15). This extra-domain now was confirmed for green algae, which also are known to include the plant-specific carbonic anhydrase subunits (11).

Topology of Carbonic Anhydrase Subunits of Arabidopsis Complex I—Based on carbonate extraction, CA2 is anchored to the inner mitochondrial membrane by hydrophobic interaction. Protease protection experiments reveal anchoring of the protein by a region close to one of its termini. Because the N-terminal two-thirds of the protein has clear homology to the hydrophilic carbonic anhydrase domain of the prototype γ-carbonic anhydrase from *M. thermophila*, anchoring of CA2 to the membrane most likely is mediated by its C terminus. Strikingly, compared with the *M. thermophila* enzyme, CA1, CA2, and CA3 have a C-terminal extension of 27–47 amino acids (supplementary Fig. S1).

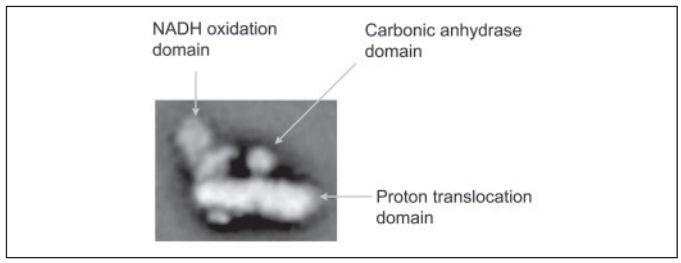

FIGURE 7. **Domain structure of plant complex I.**

The 2-kDa segment of CA2 accessible by proteolysis during protease protection experiments probably is represented by this C-terminal extension, which is exposed to the mitochondrial intermembrane space. In contrast, the main part of the 30-kDa CA2 protein is not degraded during protease protection experiments and seems to form a carbonic anhydrase domain of about 25 kDa on the matrix side of the inner membrane.

We speculate that the spherical extra-domain of Arabidopsis complex I represents the plant-specific carbonic anhydrase subunits of this complex. The extra-domain has a diameter of about 6 nm, corresponding to a molecular mass of ~75 kDa, which could include three carbonic anhydrase domains. X-ray crystallography of the γ-carbonic anhydrase of the Archeon *M. thermophila* has revealed a homotrimeric structure of this enzyme with dimensions of 65 × 65 × 70 Å (36), compatible to the diameter of the extra-domain found by EM. The γ-carbonic anhydrase subunits of Arabidopsis have a rather high homology to the γ-carbonic anhydrase of *M. thermophila* and even show immune cross-reactivity to antiserum raised against this γ-CA (12). It therefore is very likely that the complex I-integrated γ-carbonic anhydrase domain of Arabidopsis also forms a trimeric structure. However, information on the precise anchoring of the CA subunits to complex I in plants has to await further structural investigation.

Upon EM analysis, the spherical extra-domain is the only visible difference between complex I from plants and other eukaryotes on the matrix side of the membrane arm of complex I. It therefore is unlikely that the CA subunits are represented by a different region of the membrane arm on its matrix surface. However, EM analysis of complex I of an Arabidopsis knock-out mutant of the gene encoding CA2 yielded a complex I particle, which is (almost) identical to the wild type (Fig. 6, *panel i*). We interpret that the absence of one of the five complex I-included CA or CAL subunits can be compensated by the remaining proteins. Indeed, cells of the Arabidopsis knock-out mutant clearly have reduced levels of complex I, which nevertheless has a normal subunit composition (14). We speculate that the CA subunits form a heterotrimeric domain and that different CA or CAL subunits can replace each other. The biological reason for the occurrence of five structurally related CA or CAL subunits within Arabidopsis complex I remains to be established.

In summary we conclude that the Arabidopsis complex I is composed of three different subcomplexes: the main matrix-exposed domain constituting the NADH oxidation activity, the membrane arm involved in proton translocation across the inner mitochondrial membrane, and the spherical extra matrix domain most likely constituting a γ-type carbonic anhydrase domain (Fig. 7).

Physiological Role of Carbonic Anhydrases in Plant Mitochondria—Although the spherical domain is attached to complex I by a very thin stalk, this extra-domain is very tightly attached to the respiratory complex and not ripped off by biochemical extractions like carbonate treatment. Interestingly, complex I from Arabidopsis has a cavity directly opposite to the point of attachment of the spherical domain. Possibly

the γ-carbonic anhydrase domain of complex I is physiologically linked to a pore-like structure within complex I involved in proton translocation. In cyanobacteria, CO_2 hydration also takes place at the cytoplasmic face of a homologous NADH dehydrogenase complex and is thought to be linked to proton translocation activity of this complex (reviewed in Badger and Price, Ref. 37). This NADH-linked carbonic anhydrase constitutes an important part of a CO_2-concentrating mechanism in cyanobacteria, which is very important for photosynthesis. In contrast, plant mitochondria have to handle excess CO_2 that is liberated during the citric acid cycle and other catabolic reactions within these organelles and additionally during photorespiration. Indirect involvement of mitochondrial CAs in photorespiration is supported by the down-regulation of CA genes when plants are cultivated in the presence of elevated CO_2 concentration (14). It will be interesting to further explore the physiological role of the complex I-integrated mitochondrial γ-carbonic anhydrases in higher plants.

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